

The University of Chicago

THE SPAWNING REACTION
AND SPAWNING INTEGRATION OF
NEREIS LIMBATA WITH EMPHASIS
UPON CHEMICAL STIMULATION

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1936

By

GRACE TOWNSEND

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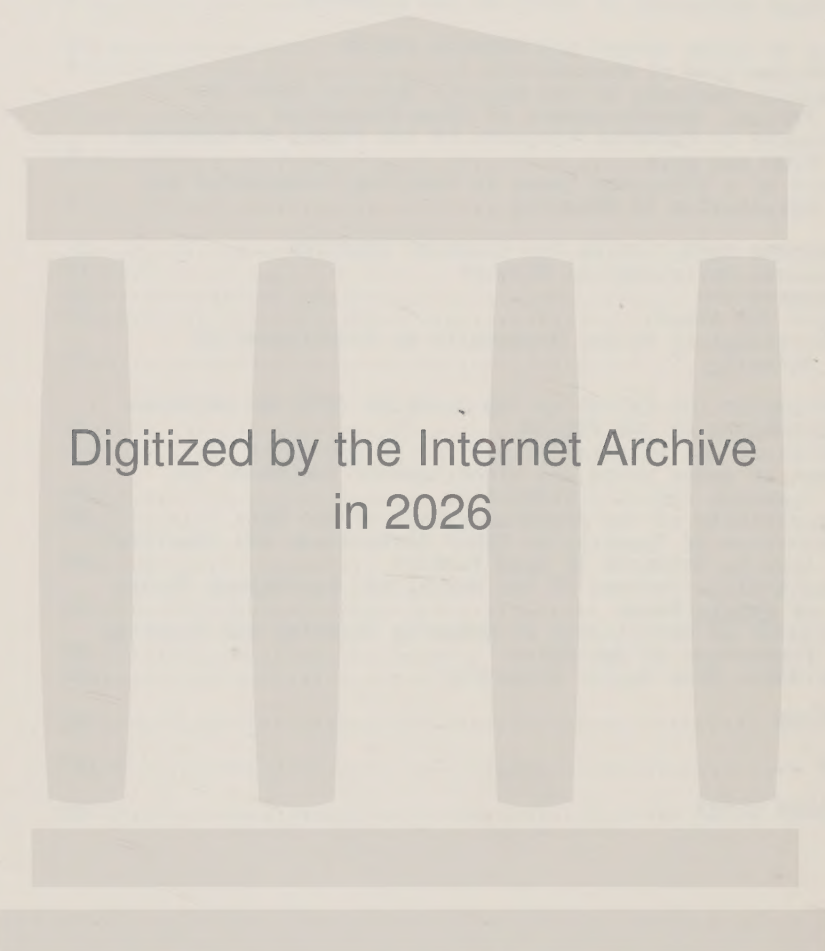


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INTRODUCTION

The spawning swarms of annelids had received attention of naturalists for two hundred years¹ before their behavior was subjected to laboratory study. In the excitement of observing that annelids may swarm in teeming thousands, that they may be used as food, and that some of them display a spectacular lunar periodicity, no attention was given to detailed studies of the swarming behavior. Even such an able investigator as Mayer in 1908 gave no fuller account of the swarming reactions of the Atlantic Palolo worm than that it swam westward, spiraling in a clockwise direction. Woodward in his final description of the spawning of the Pacific Palolo in 1907 was content to note that "the process was like an explosion" and that examination of the collapsed integument confirmed the idea of an explosion in that there were lateral rents in the body wall.

The first experimentation on the spawning reflex of a swarming annelid was that of Lillie and Just in 1913 for the species which swarms most abundantly at Woods Hole in the summer months, Nereis limbata Ehlers (= Nereis succinea Leuckart). They observed that the ripe males shed sperm in the absence of the female if placed in water in which the sexually mature female had swum, and that the presence of sperm was apparently the usual spawning stimulus for the sexually mature female. Eggs were believed to be the source of the substance which induced spawning

¹Rumphius, a Dutch naturalist, in 1705 records the swarming of the "wawo" of the Malay Archipelago for the years 1684-1694. D'Ambionische Rariteitskammer, etc., 1705, p. 51-54 (cited in Rumphius Gedenkboek. Amsterdam: J. H. de Bussy, 1902).

of the male since neither the non-sexual phase nor collapsed and spent females were capable of elaborating the substance. Moreover, since the material was present only in females with entirely ripe and fertilizable eggs, and since it was neutralized by sperm, they identified it with "fertilizin", the substance named by Lillie (1913) as essential to fertilization and initiation of development. The final spawning behavior was initiated by the presence of some substance from the female which stimulated the males to circle about the female and to spawn. In response to the shed sperm, the female was likewise induced to circle and spawn. Strikingly integrated groups of circling worms resulted, typically several males about a single female.

Since this study similar descriptions of spawning behavior¹ have been recorded for five species of nereids: Perinereis cultrifera (Herpin, 1923), and Nereis irrorata (Herpin, 1926), Nereis dumerilii (Just, 1929), and Nereis zonata and Nereis rava (Fage and Legendre, 1927). The effect of a substance from the female was investigated only for the first three of the above species. Gravier and Dantan (1928), after studying the question of lunar periodicity for 68 species of Polychaetes in the Bay of Algiers, state in a discussion of nereids that many species show characteristic circling during their spawning behavior.

The present work is an extension of the observations of Lillie and Just on N. limbata. An attempt has been made to analyze the circling response and to investigate the chemical nature of the material elaborated by the female which induces the

¹An excellent description of a contrasting spawning behavior of a swarming nereid is that of Just (1914) for Platynereis megalops. For two syllids, Herpin (1923, 1926) observed that the females did not spawn until after the males had shed sperm in the water.

male to spawn. We shall consider: (1) the muscular mechanics of circling and spawning, (2) the rôle of the sense organs and nervous system, (3) some environmental and physiological factors conditioning the spawning reaction, and (4) the nature of the substance or substances elaborated by the female worm which induce the circling and spawning of the male. The last phase will be treated in greatest detail.

I. MUSCULAR MECHANICS OF CIRCLING AND SPAWNING

The mechanics of the spawning response appears to be entirely similar in the two sexes but may be more readily observed in the female because of the slower swimming. The response is initiated by circling, to which motion is added, during active spawning, a vibratory motion of the middle segments. The complete reaction appears to involve all sets of muscles, an impression which is confirmed by analysis.

The circling tends to keep the sexes in close proximity during spawning and to result in a concentration of sex products in a relatively small compass of water. The circling may be of two types involving primarily the ventral or the dorsal longitudinal muscles, respectively. The circling initiating spawning results from a ventral curling of the anterior tip of the worm produced by contraction of the strong ventral bands of longitudinal muscles. The position of the head is the same as that assumed by the quiescent worm in initiating an undulatory respiratory movement (part of the movement described by Hempelmann, 1929, and Stolte, 1932, as "helicoidale", but here the head remains curled ventrally and guides the worm in ventrally directed circles.

After expulsion of the sex products and a rather complete collapse of the worm, the posterior portion of the epitoke curls dorsally. In this state, worms which recently circled because of a ventral curling, now circle from a dorsal curling. The curling is not a passive state but results from contraction of the dorsal longitudinal muscles and may be induced in spawned worms which are swimming normally.

A combination of these opposed contractions, tends to shorten the worm, to place the contents under pressure, and to develop diagonal strains upon the inter-parapodial region which favor rupture or opening of pores.

Two additional muscle actions may be analyzed in non-swimming worms mechanically induced to spawn. The first concerns the mechanics of the vibratory motion and is most clearly observable in a male worm when removed from water while swimming and at once stimulated to spawn. It consists of lateral sinusoidal movements of the middle segments (figured by Buddenbrock, 1930, p. 312) coördinated with vigorous paddle-like movements of the parapodia. Such movements of the parapodia undoubtedly serve to dislodge the sex elements from within the bases of the parapodia where they are densely packed. The coördination of lateral bending and parapodial beating produce repeated lateral inter-parapodial tensions which aid in the expulsion of the sex elements. The second supplementary component is most readily observed in the female and consists of peristaltic contractions of the circular muscles beginning posteriorly and progressing anteriorly. In the swimming worms, the spawning similarly progresses forward from the posterior tip.

II. ROLE OF SENSE ORGANS AND NERVOUS SYSTEM

1. Coordination of Musculature

If the cerebral ganglion of the male is destroyed by cauterization or cutting, or if the brain is isolated by cutting of the circumpharyngeal connectives, the worm may be stimulated to more rapid swimming by female charged water but no circling or orientation takes place. If only one circumpharyngeal connective is cut, a modified orientation is still possible. If the suboesophageal ganglion is destroyed, the worm can no longer swim but progresses by vibratory movements along the bottom of the dish. Hamaker (1898) in his detailed study of the nervous system of N. virens found all of the giant fibers pass through the suboesophageal ganglion before reaching their ganglion cells. In the earthworm the giant fibers are concerned with the rapid shortening reaction (Bovard, 1918-a; Stough, 1930; Holst, 1932). Bovard (1918-b) found transmission along these fibers in the earthworm is fifty times that of impulses functional in crawling. The sexual phase of N. limbata is approximately one-third shorter in body length in the swimming than in the quiescent state. The shortened state and the extreme rapidity of the movements of swimming as contrasted to those of crawling, as well as the inconclusive evidence of loss of function after mutilation of the suboesophageal region including the tracts, suggests that the giant fibers may play a significant rôle in swimming.

The destruction of the cerebral ganglion only was tried in the female. In the five worms tested, it prevented circling and the normal spawning reaction in the same manner as in the male.

2. Sensory Response of the Male to Material from the Female. Metamorphosis of Chemo-Receptors

Copeland and Wieman (1924) found that the food chemo-receptors of N. virens are concentrated anteriorly especially upon the tentacles and palpi. Clipping of tentacles and palpi of male N. limbata did not affect the sensitivity to egg-water. Furthermore by placing the worm in glass tubing it was ascertained that very little sensitivity to egg-water exists anteriorly. Moreover, greatest sensitivity to egg-water was observed to be on the highly metamorphosed epitoke, especially just anterior to the attenuated small posterior segments.¹ Only epitokous fragments of cut worms may be stimulated to movements characteristic of spawning.

The non-sexual phase is only slightly if at all sensitive to the spawning inducing compounds and the sexual phase shows no increased sensitivity to some twenty chemicals as tested (see Section IV) except for compounds inducing spawning. This indicates that a metamorphosis or development of the chemo-receptors sensitive to egg-water occurs at sexual maturity.

In nature, after brief spawning the males rapidly swim away as soon as the female has completed spawning, rarely discharging all of the sex products in response to one stimulation. Experimentally subjected to repeated short intervals of stimulation they may execute forty successive spawning responses. The short reaction time, the localization of the sensory areas, and the continuation of spawning typically only in the presence of a

¹Gravier and Dantan (1928) have noted that the loss of the posterior portion of certain nereids results in a loss of circling and orientation to the opposite sex and interpret this as evidence that a chemical sense functional in orientation may be located in the pygial organ. In N. limbata the pygial organ in the male is usually lost very soon after initiation of swimming and the loss is not accompanied by any discernible change in chemical sensitivity unless a very large portion of the epitoke is lost as well.

stimulus indicate that the spawning of the males is a sensory and nervous response. Nevertheless, as will be considered below, males subjected to repeated stimulation may sometimes continue circling for an extended period of time even though rinsed several times in freshly drawn sea-water. Addition of sperm hastened the return to normal swimming.

3. Sensory or Hormonal Response of the Female to Material from the Male

Contrasted to the typical nervous response of the male, the spawning of the female exhibits traits typical of hormonal control. Once initiated, spawning continues to completion and the circling continues for some time following, even though all stimulus may be removed by placing the female in freshly drawn sea-water. Moreover, the response to sperm is not an immediate one but involves a short or extended delay.

Galtsoff (1930), studying the spawning of the oyster, found males respond immediately to the presence of eggs in the water while the females respond to sperm only after a lapse of many minutes. He considered that the male response was of a sensory nature while the female response was hormonal. Although the female worm possesses a similar delayed response and further hormonal characters above enumerated, there are certain limiting considerations. (1) By reason of the complexity of the muscular coordination, nerve function necessarily plays a part. (2) The period of delay is greatly shortened by a preparatory half-hour or hour of swimming. To a less marked degree the reaction time of the male is influenced by an initial period of swimming. In the physiological state following spawning the female may respond in almost as short an interval of time as the males. (3) The prolongation of the response in the female shows a tendency to vary with the length

of period of stimulation inducing it. Since both sperm and a sperm and egg mixture are stimulating to the female, the stimulus in nature is typically a long one. Following spawning, females may be experimentally subjected to a stimulus of short duration and exhibit a short period of response. Finally it is remembered that a prolonged response, such as is typical for the female, may sometimes be artificially induced in the male by long-continued stimulation. In nature, since the sperm-egg mixture does not remain stimulating to the male, the period of stimulation is typically brief.

In view of the growing evidence of the closeness of relationship of nervous and hormone action (Parker, 1932; Bain, 1933; Cannon, 1933), it is possible that the behavior of the female may merely indicate that a long-continued nervous response may become hormonal. The spontaneous circling of the males after prolonged stimulation could thus be explained as well as the prolonged reaction of the female following an initial prolonged stimulation in the presence of a refractory physiological state. By this interpretation both male and female responses would be essentially nervous and quite similar except for an initial inhibitory physiological state on the part of the female which prevented response until an accumulation of nerve secretion occurred. Response after cessation of stimulation on the part of the female would result from the accumulation of nerve secretion. The male by reason of a sensitive physiological state would exhibit immediate response involving no such prolongation of nerve secretion.

4. Role of a Vibratory Sense in Circling Integration and Orientation in Swimming

If the non-sexual worm is forced to swim in a finger-bowl, it traverses the bowl freely in any direction; the heteronereis

phase, however, orients and tends to swim at a uniform distance from the sides of the bowl. A very characteristic pattern of swimming results which may be maintained for hours if the worms are left undisturbed. They will turn from a solid object placed in their path without striking it. If the object is slender they may show some tendency to circle about it. This orienting sense undoubtedly plays some role in the circling of the male about the female.¹ If a drop of egg-water which contains carbon is added the male may be seen to turn toward the greatest concentration of the egg-water and even to swim through the drop. If only the chemical sense operated in circling integration, the males would undoubtedly come into collision with the females in turning toward the source of stimulation.

Sight cannot be the controlling sense in this reaction for orientation takes place equally well in varying degrees of illumination. Also worms with eyespots cauterized still exhibit characteristic orientation.

If over-stimulated continuously circling males are introduced into a bowl containing several males swimming oriented to the sides of the bowl, the latter orient to the introduced spiraling worms and swim in a manner indistinguishable from them. A spectacular pattern of circling paths results which will be maintained over a long period of time. (See Plate I.) Removal of the introduced males results in a return of the remaining worms to the typical peripheral "around the bowl" swimming.

The females possess a similar but less sensitive orientation sense and collide relatively frequently with objects.

¹In Platynereis megalops, a species of contrasting breeding behavior in which the male wraps itself about the female, the males frequently collide with the sides of a containing vessel and often swim against objects in a manner suggesting a positive orientation to them.

The dorsal and ventral parapodial cirri were clipped from the epitokes of four anesthetized sexually mature males with iridectomy scissors. Three of the four worms, together with their controls, recovered but in contrast to the controls had lost much of their tendency to swim oriented to the sides of a bowl and traversed all parts as do swimming, non-sexual worms. At metamorphosis, the parapodial cirri of the epitoke become greatly elongated and to a greater extent in the males than in the females.

Langdon (1900) found a concentration of groups of sensory cilia on the parapodia of N. virens. Ariens-Kappers and Droguever-Fortuyn (1920) conclude that the sensory fibers are probably limited to the dorsal and ventral cirri. Stolte (1932) reports sensory cilia on the dorsal parapodial cirri of Glycera and notes that these are not stimulated by chemicals or by touch. However, he was able to stimulate them by causing them to oscillate ever so slightly. He named this sense the "Erschütterung" sense but did not attempt to explain its normal function.

III. FACTORS CONDITIONING THE SPAWNING REACTION

1. General Environmental Factors

The general factors related to swarming of N. limbata, such as, season, lunar periodicity, and the diurnal rhythm have been described by Lillie and Just. Regarding these phases, the present work includes only some observations regarding light and tactile stimuli which may relate to the diurnal rhythm.

If males in a shallow layer of sand in a lighted place were placed in darkness they were stimulated to swim within approximately a half-hour interval. They could again be made quiet by exposure to light, the rate varying from a few minutes with light of the intensity of photo-flood bulbs to a few hours with a dim ceiling illumination. With the use of intense illumination, the quiescence and swimming could be alternated every two or three hours.

Intense illumination apparently induced quiescence by increasing the number of encounters with the bottom. Under the influence of photo-flood bulbs the worms in the course of their typical rapid swimming would make excursions between top and bottom and if in some excursion they lodged themselves sufficiently in the sand, the worms responded by crawling and burrowing. In all cases the tactile stimulus appeared to play an important role in the final settling. If swimming worms were placed in smooth containers without sand, they frequently swam more than twenty-four hours or until entirely weakened. Contrastingly, if sea lettuce was so placed that the worms encountered it in

their swimming, they lodged themselves, began a crawling response, became quiescent and secreted a case of slime.

Thus it would appear probable that response to tactile stimuli plays a role supplementary to light intensity in the normal diurnal rhythm. In a region of depth and current, as in Great Harbor at Woods Hole, swarming may continue until eleven o'clock on the same evenings upon which, in the relatively shallow quiet waters of the Eel Pond, swarming had ceased by half past nine. This may be explained by the fewer tactile stimuli encountered in the greater depth, and also by a tendency of the current to free insecurely lodged worms.

2. Temperature

Within a range between 14° and 26° C. a rise of temperature increases the rate of swimming and sensitivity of the worms to chemicals. However, the effect is somewhat conditioned by their past history. Worms kept in an icebox at 11° for days and worms collected from autumn waters (sometimes as low as 16° as contrasted with the 20° and 21° of midsummer) manifested optimum spawning responses in colder water than worms collected and kept at higher temperatures. A rising metabolism was apparently characteristic of the worms achieving the physiological state for spawning. Even with the induction of swimming, a physiological state adapted to spawning could not be attained with too low temperature.

3. Acid and Alkali

Within the range of non-toxic tolerance (pH 6.0-9.0) dilute acid increased and dilute alkali decreased sensitivity. The effects were the same whether the acid or alkali were added to the sea-water in which the worms were swimming or added to the egg-water or other solution used in stimulating the male worms. Whether the effect

was a direct one involving the sensory end organs, or an indirect one involving the egg-water and chemicals, was not determined by any of the several experiments devised.

4. Physiological State, Especially as Conditioned by Swimming

Dehorne (1922, 1926) reports that during sexual metamorphosis of nereids the general body muscles are transformed from smooth to striated muscles. During the processes a substance inducing contraction appears and the worms are greatly shortened. The average length of N. limbata in the sexual phase is shorter than the non-sexual phase and becomes about one-third shorter during the first five or ten minutes of swimming. A quiescent sexually mature worm cannot be induced to spawn in a normal manner without a preliminary period of swimming.

After a period of quiescence both males and females may remain without response when subjected to material potent to induce spawning of the swimming worms. Sperm may be added to bowls containing females quiescent in sea lettuce until the water is milky and the worms, unless extraneously stimulated to activity, will remain over night without spawning. If dislodged from sea lettuce the following morning, after a short period of swimming, a female may be induced to spawn by the sperm which had been present all night.

Males in a quiescent state may be similarly treated with egg-water without evoking response. However, a strong dosing of the area of greatest sensitivity may induce a slight movement; if stimulation is repeated, the worm may become active, and apparently in so doing, increase its chemical sensitivity and begin to swim. Upon swimming, sensitivity rapidly increases and spawning soon follows. Several male and female worms may frequently spend the night

in the same fragment of sea lettuce without reciprocal stimulation or spawning.

In the third place, during swimming there is the elaboration of material by each sex respectively stimulating to the opposite sex. Males shed their sperm naturally only while swimming and sperm induces the spawning of the female. Similarly, the female gives off the material which constitutes the normal stimulus inducing spawning of the male while swimming. Even after days of quiescence, the surrounding water was not charged with the material elaborated in swimming, and some observations indicated that such material was rapidly diminished by the presence of a quiescent worm.

If the females were kept quiescent for several days, as was possible in the icebox, the spawning inducing material contained in them was diminished in quantity even though the vigor of the worms was maintained. Furthermore, if these worms were caused to swim, the quantity of spawning inducing material present was replenished. This increase in quantity was indicated by three types of observations. (1) The amount of material elaborated in the swimming of the icebox aged females was reckoned and added to that freed by the same worms in spawning, and the total amount compared with the amount freed from control¹ worms which had either remained

¹The minimum concentration of female material necessary for one drop (1/20 cc.) to induce spawning of the male was considered to be the minimum effective concentration and to possess a strength of "unit" potency. The quantity of material in a given volume was assayed by determining the maximum dilutions possible to yield unit potency and recorded as the cc. volume resulting from the dilutions, i.e., the maximum volume possessing unit potency. The quantity of material in the water in which the worms were swimming was determined at intervals and the water changed after each determination. The quantity of material freed in spawning for the swimming and control worms was determined after placing the females in freshly drawn sea-water and artificially inducing them to spawn.

quiescent since being taken from the icebox, or were freshly taken from the icebox. A summary of the two experiments of this type are given in Table I. (2) The rate of elaboration of spawning inducing material elaborated by icebox aged females after 2, 4, 6, etc. hours of swimming was correlated with the number of hours the worms had previously swum at the time the rate was determined. Graph I records the results of these observations. (3) The amount of spawning inducing material freed by icebox aged females upon spawning, as separate from that elaborated in swimming, was correlated with the number of hours the worms had swum previous to spawning. Graph II records the results of these determinations.

By studying Table I, it may be seen that the total amount of spawning inducing material elaborated in swimming and spawning by the experimental active worms was at least three to four times the amount freed by the control quiescent worms upon being mechanically induced to spawn. Student's formula for testing statistical significance gives a P value of 0.0008 for each of the two experiments, i.e., the probability of chance giving the same difference in either experiment is eight in ten thousand. Moreover, in each of the experiments, the amount of material elaborated in swimming or the amount freed in spawning is alone more than the amount freed by the quiescent control worms.

Graph I is compiled from four series of observations. The use of combined series was necessary because many females spawn spontaneously before they have swum even eighteen hours. Further observations not represented indicated that fresh worms reached a maximum rate of elaboration in a relatively short time. This graph shows that these icebox aged females did not attain a maximum elaboration of spawning inducing material until after eight to fourteen hours of swimming. The rate subsequently de-

TABLE I
COMPARISON OF AMOUNT OF MALE SPawning INDUCING MATERIAL PRESENT
IN ACTIVE WORMS AS COMPARED TO QUIESCENT WORMS

EXPERIMENTAL--ACTIVE WORMS					CONTROL--QUIESCENT WORMS	
Size in cm. Length	Hours of Swimming	Volume in cc. Calculated to Give Minimum Effective Stimulation			Size in cm. Length	Volume in cc. Cal- culated to Give Minimum Effective Stimulation. Freed with Eggs
		(a) Elaborated in Swimming	(b) Freed with Eggs in Spawning	Total (a) and (b)		
2.6	2	600	240	240	2.8	450
2.5	2	600	120	720	2.4	240
2.4	2	500	180	680	2.4	90
2.4	2	300	660	960	2.4	60
1.9	2	300	630	930	1.9	75
1.7	2	300	180	480	1.8	75
1.6	2	200	195	385	1.7	75
1.6	2	30	180	210	1.5	90
1.4	2	30	180	210	1.2	30
Mean with S.E. =		318 ± 71.9	285 ± 71	603 ± 97	(P=.9996)	132 ± 44.7*
2.3	4	1500	200	1700	2.3	1050
2.6	12	1500	300	1800	2.3	1050
2.3	4	1500	1200	2700	2.3	900
2.3	8	1000	2000	3000	2.3	900
2.6	8	100	2000	2100	2.3	900
1.4	12	4000	1600	5600	2.4	1050
2.3	4	65	3300	3365	2.3	1250
2.3	24	2637	150	2787	2.3	1050
2.3	18	2180	2250	4430	2.3	900
Mean with S.E. =		1609 ± 365	1444 ± 419	3054 ± 134	(P=.9996)	1006 ± 46.0*

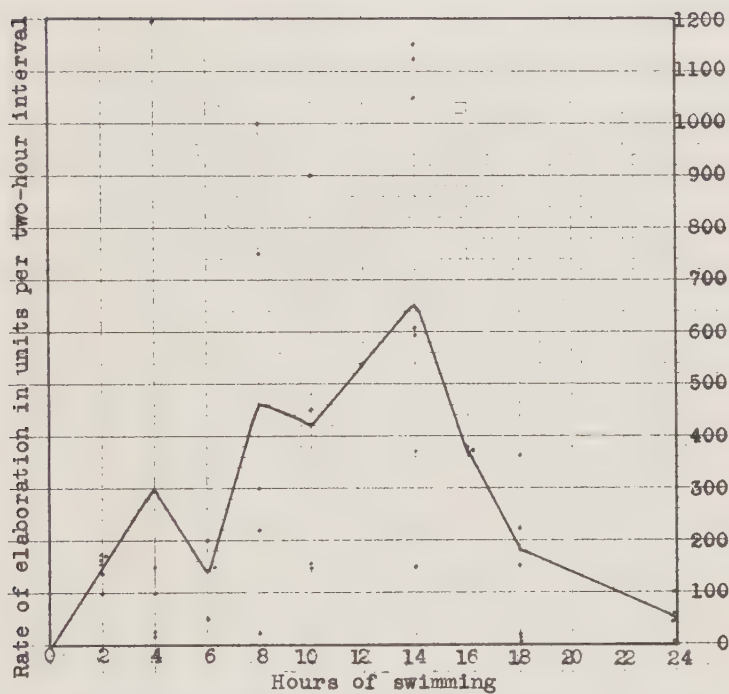
*The control worms of the first series were left at room temperature during the interval of the experiment. The control worms of the second series were kept at icebox temperature.

clined, probably because of fatigue and a weakened state of the worms but possibly because of depletion of the source material. The correlation coefficient of the elaboration rate and time of swimming calculated for the first fourteen hours of swimming is $0.48 \pm \text{S.E. } 0.14$.

Graph II involves a compilation of observations of 60 icebox aged females and is arranged to show the relationship between the amount of materials freed in spawning to the length of interval of previous swimming after the quiescence in the icebox. Only instances are included in which the worms were in freshly drawn sea-water at the time of spawning. The correlation coefficient of the amount of material and time of swimming is $0.79 \pm \text{S.E. } 0.048$ for the period of the first twelve hours. A comparison with the preceding graph suggests a high correlation between the rate of elaboration of spawning inducing material and the amount actually freed in spawning for corresponding time intervals after initiation of swimming. It may be noted that no duplicate determinations are represented in the two graphs.

This series of observations indicate that the amount of free spawning inducing material in the icebox aged female is greatly increased during swimming.

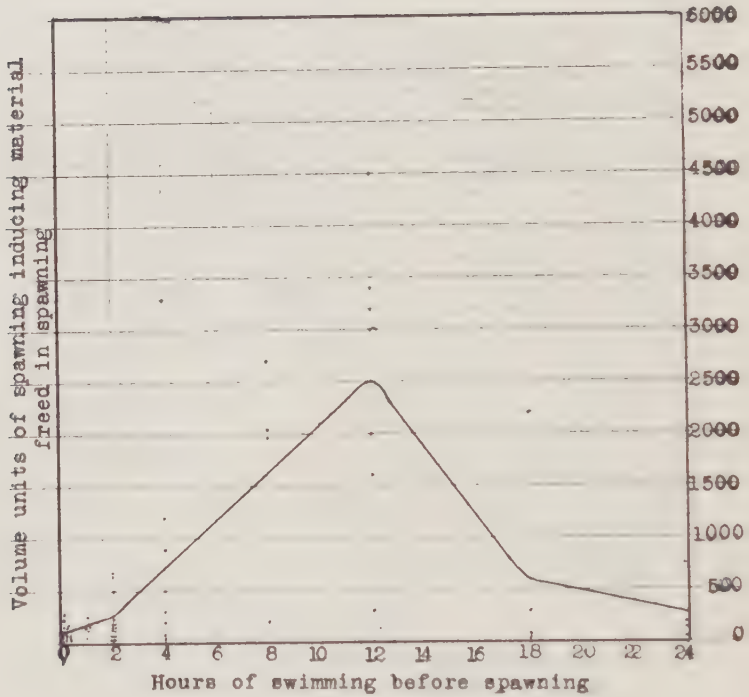
GRAPH I
RELATION OF RATE OF ELABORATION OF MALE SPAWNING
INDUCING MATERIAL TO TIME OF SWIMMING FOR
ICEBOX AGED FEMALES



The dots indicate the rate per two-hour interval for individual works, and the line shows the mean rate.

GRAPH II

RELATION OF AMOUNT OF SPAWNING INDUCING MATERIAL FREED WITH
EGGS TO LENGTH OF TIME OF SWIMMING BEFORE SPAWNING
FOR ICEBOX AGED FEMALES



The dots indicate the amount of material freed by individual worms in spawning and the line, the mean at successive intervals of time.

IV. CONCERNING THE NATURE OF THE SPAWNING INDUCING MATERIAL ELABORATED BY THE FEMALE

Two methods of investigation concerning the nature of this material were tried: (1) a chemical analysis of egg-water as representing a source rich in the spawning inducing material; and (2) a direct testing of various compounds for any influence on the physiology of spawning. Obviously the second method could only furnish suggestions for further investigation.

1. Chemical and Physical Properties of Egg-Water

Egg-water of a syrupy consistency was subjected to various protein tests. Some slight yellow coloration but no precipitate was obtained with the xanthoproteic test, some slight precipitate but no violaceous color upon heating was obtained with Millon's reagent, an indistinct blue color was obtained with the ninhydrin reagent, no distinct difference in color of sea-water and egg-water could be determined after application of the biuret test. In all cases the tests were negative or uncertain. These findings are in agreement with those of Lillie (1919) and Glaser (1914).

During the period of intense interest in the constituents of egg-water following Lillie's discovery that a substance essential to fertilization of eggs was given off into the surrounding water (Lillie, 1913), a technique reported by Robertson (1912) in a study on mammalian blood sera was applied to egg-water by Woodward (1914) in studying "fertilizin."¹ Certain principles of

¹Since the author has observed that a concentration of sea-salts does not induce spawning, the limitations of Woodward's method, as pointed out by Just, does not interfere with the present study.

the method were employed to supplement the vague information gained from the protein tests.

Ammonium sulfate in saturated solution precipitates all proteins and proteoses but not peptones and peptids. Organic compounds of the complexity of the latter as well as many inorganic compounds may be precipitated by alcohol and acetone. Hence if the spawning inducing material involved complex protein molecules, it would be precipitated by a saturated solution of ammonium sulfate. If it involved only relatively simple molecules it should be precipitated by acetone and not by saturated ammonium sulfate. The latter was found to be the case.

A concentrated solution of egg-water obtained from the eggs of forty female worms yielded a precipitate when saturated with ammonium sulfate, but the precipitate contained no spawning inducing principle. A similar concentrated solution of egg-water filtered through a Berkefeld filter to remove very large molecules and subsequently treated with four volumes of acetone, yielded a precipitate containing a high concentration of the spawning inducing principle. The precipitate was too impure for study of physical properties. That this material was not precipitated by saturated ammonium sulfate but was precipitated by acetone, indicated that relatively simple molecules were involved. Dialysis through collodion membranes was tested.

Egg-water possessing a high concentration of the material was placed in each of four three-ply collodion membrane bags. Within an interval of five to ten minutes the spawning inducing material could be detected in the water surrounding each of the membranes. This indicated a rapid rate of diffusion through a membrane of fine pores. This would be only possible for molecules of small size.

Boiling egg-water for from five to sixty minutes gradually destroyed the spawning inducing material; the time of destruction depended upon the original concentration. Aging of egg-water for a few hours or days similarly resulted in a gradual destruction of its spawning inducing property. An acidity of pH 6.0 decidedly increased the stability; a high acidity, obtained by addition of one part of concentrated HCl to fifty parts egg-water, preserved the material indefinitely. Addition of dilute NaOH to give an alkalinity of pH 9.0 or above facilitated destruction.

Proteolytic digestion provided a further means of investigating the nature of the molecules under investigation. Carbohydrates would seem less likely to be derived from eggs or female worms than nitrogenous substances and any lipoids would not be precipitated by acetone. If the physiological property of the material inducing spawning depended upon a molecular configuration involving amino acid linkages, it should be destroyed by an appropriate proteolytic digestion. However, in order to test the effect of proteolytic enzymes, the preparations utilized should not in themselves possess a physiological action which would conceal or eliminate the spawning response of the worms. Trypsin, pepsin, and papain were successively tested.

Trypsin in dilute solution was found to have no effect on the worms, and, when mixed with a solution of the spawning inducing material, to inactivate it completely. However, it was soon demonstrated that the action of the preparations was not through enzymatic splitting of molecules. Boiling the trypsin preparation for ten minutes, which would destroy all of its enzymatic properties, did not affect its capacity for inactivation. Trypsin is amphoteric and is frequently used as an adsorbent in the purification of other enzymes. It is possible that the inactivation was

effected by adsorption.

Preliminary tests of pepsin and papain preparations showed that, in 2 to 10 per cent solutions, both caused typical circling and shedding of sperm. As this effect took place in sea-water and pepsin is only active in an acid medium, it was impossible that the effect could have been due to enzymatic action, at least with pepsin. Furthermore, boiling did not destroy the spawning inducing property of either the pepsin or papain preparations. These observations suggested that some substance associated with the samples of pepsin and papain evoked the spawning reaction. Proteins and protein derivatives would be expected to be the most abundant impurities.

Accordingly 5 per cent solutions of "Bacterio-Peptide" (unknown source), beef extract (Leibig), and "Aminoids" (Arlington Chemical Co.) were prepared. Although all evoked the spawning reaction to some degree, the preparation of "Aminoids" was the most effective. This preparation is a biuret-free (i.e. without peptid linkage) mixture of amino acids. It therefore seemed possible that a single amino acid might induce spawning.

2. Test of Amino Acids and Miscellaneous Compounds for Spawning Inducing Properties

One per cent solutions of seventeen amino acids were prepared, and each was tested on several males in each of three fingerbowls. The minimum effective concentration of any substance was considered to be that necessary for a one-drop quantity (1/20 cc.) to induce spawning when the drop was introduced into a vessel of sea-water containing swimming male worms (see footnote, p. 15). For uniformity, the same volume of water and the same kind of vessel was used throughout any experiment. However, uniformity of these factors was probably of no importance, as the

worms appeared to respond to a gradient of concentration between any spawning inducing substance and sea-water rather than to an absolute concentration. A considerable quantity of potent material could be added without inducing spawning if mixed uniformly through the sea-water. The experimental results obtained in testing the series of amino acids are recorded in Table II.

The table shows that only cystine and cysteine of the amino acids tested were strikingly effective in inducing spawning. Cystine and cysteine are distinguished from other amino acids in possessing sulfur in the form of the oxidized and unoxidized sulphydryl radical, respectively. Other amino acids even if slightly effective were stimulating only in such high concentrations as to suggest cystine or cysteine impurity.

The nitro-prusside test¹ for sulphydryl radical (or for some reducing substance and the sulphydryl is the one most abundant in proteins), sensitive to 15-20 parts in a million, was applied to 1 per cent or saturated solutions of each of the amino acids tested. All of the preparations capable of inducing the spawning reaction gave positive nitro-prusside tests. The hypothesis that the spawning inducing property of certain of the preparations was due to cystine impurities gained additional plausibility when a sample of leucine lost most of its stimulating properties upon purification by recrystallization. Such could not have been the case if leucine, per se, were the active principle. Bacterio-Peptide, beef extract, Aminoids, and pepsin and papain preparations, which as noted above had been ascertained to

¹Walker's modification of the nitro-prusside test using NaCN in place of NH₄OH was employed (Walker 1925). Where suitable a further modification of addition of ZnCl₂ was utilized (Okuda and Nishijima 1926). As zinc may give a color reaction with certain amino acids, this modification was not used in the above series of tests.

TABLE II

EFFECT OF VARIOUS AMINO ACID PREPARATIONS ON SEXUALLY
MATURE N. LIMBATA, TOGETHER WITH THE RESULTS OF
TESTING EACH PREPARATION FOR THE SH GROUP

Amino Acid 1% or Satu- rated Sol.	Source	Nitro- Prus- side Test	Spawning Response	Effective Concen- tration	Treatment and Effect
Glycine....	E.K.	+	+	3%	3 d. spawning
Alanine....	A.H.T.	+	+	4%	4 d. spawning
α -Amino butyric....	E.K.	+	+	1%	1-2 d. spawning
α -Amino valeric...	E.K.	+	+	2%	2 d. spawning
α -Amino isovaleric	E.K.	-	-?		1-10 d. spawning
Leucine....	Koch	++	+	1%	1 d. spawning
Isoleucine.	E.K.	+	+	3.5%	3-4 d. spawning
Aspartic acid.....	Unknown	-	-		10 d. tetany
Glutamic- HCl.....	E.K.	++	-		1-10 d. no effect
Hydroxy- glutamic..	Unknown	-	-		1-10 d. no effect
Phenyl alanine....	E.K.	-	-?		1-10 d. stimulating
Tyrosine...	Koch	-	-		1-10 d. no effect
Arginine- HCl.....	E.K.	+	-?		1-10 d. stimulating
L-Proline..	E.K.	-	-		1-10 d. no effect
Tryptophane	Koch	-	-		1-10 d. no effect
Histidine..	Koch	-	-		10 d. slightly toxic
Cystine....	E.K.	++++	++++	0.0028%	1 d. 1/350 dil. spawning
Cysteine- HCl.....	E.K.	++++	+++	0.14%	1 d. 1/7 dil. spawning

- = negative test or no re-
sponse
+ = positive test or posi-
tive response. Number
of crosses indicate
intensity
"tetany" = worms set in
sinusoidal curves sink to

bottom of bowl.
E.K. = Eastman Kodak Company
A.H.T. = Arthur H. Thompson Co.
Koch = gift of Dr. F. Koch, pre-
pared in University of
Chicago Physiological
Chemistry Department.

induce spawning, all gave strongly positive nitro-prusside tests.

These observations suggest that the characteristic spawning reaction of the male may be induced artificially only in the presence of certain compounds containing the sulphhydryl radical. However, the converse relationship is not true. Taurine, which contains the sulphhydryl radical and is thought to be one of the first decomposition compounds of cystine in its oxidation in the body;¹ thioglycollic acid, which is frequently used as a source of the sulphhydryl radical in growth experiments, and thioacetic acid did not induce spawning.

Other compounds possessing partial molecular configuration or radicals in common with cystine, as well as compounds of distinctive physiological properties were tested: propionic acid as possessing a fatty acid chain in common with cystine, ammonium sulfate as containing an ammonium group of properties similar to the amino group of amino acids, sodium thiosulfate as representing a reducing agent containing sulfur, ferrous iodide as representing an extreme reducing agent, iodine and butyric acid as representing parthenogenetic agents, acetyl choline and adrenalin as representing compounds affecting muscles, insulin and thyroid preparations as representing compounds affecting metabolism, etc. None of the compounds tested in this survey gave even the slightest indication of any spawning inducing property.

Finally and most logically glutathione, the compound in which cysteine and cystine occur in living tissues, was tested. This compound was from six to thirty-five times as effective as cystine solutions if the comparison was made with reference to molar concentrations, and three to sixteen times as effective if

¹A. P. Mathews, Physiological Chemistry (New York, 1929), p. 866.

the comparison was based on gram weight. Freshly prepared solutions of glutathione 1:100,000, or more commonly 1:400,000 (2.5 to 10 parts per million) added in one drop quantity to a vessel of sea-water containing swimming males induced the characteristic spawning reaction. Unusually sensitive worms sometimes responded to a single drop of a dilution of 1:1,000,000 or even 1:2,000,000.

As a final consideration, an attempt was made to find whether the reduced or oxidized state of sulphhydryl compounds influenced their power to induce spawning. The commercial form of thioacetic acid, of thioglycollic acid, and of glutathione, is the SH or reduced form. Complete oxidation of these compounds, under conditions described by Dixon and Tunnicliffe (1923) and Mason (1931) did not change their power to induce spawning. However, since some oxidation of sulphhydryl compounds would almost certainly occur in the process of dilution and the subsequent addition of sea-water involved in the testing (within the pH range not toxic to worms) there is the possibility that the oxidized form is the only form of glutathione and sulphhydryl compounds effective in the induction of spawning.¹

3. Specificity of the Chemo-Receptors of the Male

Cystine from different chemical supply houses was not equally effective; cystine from Eimer and Amend was more potent than Pfanstiehl's or Eastman's. Color tests (Sullivan's 1926, and nitro-prusside) did not indicate any difference in the chemical

¹The commercial form of cysteine is cysteine-hydrochloride and is of an acid toxicity to interfere with spawning. Since any acidity less than pH 3.5 permits oxidation of cysteine to cystine and since any acidity greater than pH 6.0 is toxic to the worms, no suitable technique for testing the effects of cysteine apart from cystine was devised. Commercial cystine and cystine prepared from cysteine, are more effective in inducing spawning than the acid solutions of cysteine hydrochloride.

purity of the samples.

Hoffman and Gortner (1922) showed that cystine exists in isomeric forms and that l-cystine, the form which occurs in nature, is gradually transformed to a less active form by boiling. If the mode of preparation of cystine employed by the various chemical companies is different, the proportion of l-cystine might be expected to differ. Each of the chemical companies obligingly described its own method of preparation and gave the typical optical property of its own product. The preparation differed, but not sufficiently to account for the observed disparity.

The rotatory properties of the preparations at hand were determined directly with reference to the mercury green line, using 2 per cent solutions dissolved in 5 per cent hydrochloric acid as follows: Eimer and Amend, -137.5; Pfanstiehl, -122.5; Eastman Kodak, -120.1. Typical comparable concentrations as to physiological effectiveness were: Eimer and Amend, 1:55,000; Pfanstiehl, 1:45,000; and Eastman Kodak, 1:40,000.

By courtesy of Dr. V. Du Vigneaud, samples of laevo-, dextro-, racemic and meso-cystines were obtained. Typical comparable concentrations were: laevo-cystine, 1:60,000; dextro-cystine, 1:5,000; racemic cystine, 1:35,000; and meso-cystine, 1:5,000. Hence the worms were about twelve times as sensitive to the laevo-cystine as to the dextro- or meso-cystine preparations.

Males of N. limbata, at the time of sexual maturity, possess a special sensitivity to a characteristic molecular configuration as represented in glutathione, cysteine, and cystine, and the sensitivity is sufficiently specific to differentiate between a right and left configuration of the molecule. All of the chemically active radicals making up these compounds when tested in other configurations, as the sulphydryl group in thioacetic

and thioglycollic, or the carboxyl group in the fatty acids, or the amino and carboxyl amino acids, were not effective.

The chemical sensitivity of the posterior segments of several N. limbata in both sexual and non-sexual phases and the non-sexual phase of N. virens and Podarke was tested. To a large extent the same series of compounds (amino acids, fatty acids, and sulphhydryl compounds) were employed. None of the non-sexual phases showed any marked sensitivity to egg-water or 1:1,000 or 1:10,000 glutathione.¹ Only the sexual phase of N. limbata showed any special sensitivity to these substances. The response or lack of response of all of the worms to other compounds than glutathione and egg-water was remarkably similar.

4. Induction of Spawning by Crude Glutathione and Inactivation by Extracts of Aged Tissues

Since the physiological properties of cystine had been found to be affected by modes of purification in the laboratory, an attempt was made to test crude aqueous extracts of glutathione (Hopkins, 1921; Tunnicliffe, 1925), along with various other materials. Such extracts were made from beef liver, round steak, kidney, and ham (from a butcher shop), various tissues from marine animals, and various tissues from a freshly killed cat. The results are recorded in Tables III and IV.

In accord with the well known fact that glutathione is present in almost all investigated living tissues, an approximately saturated aqueous extract from almost any fresh tissue was effective in inducing spawning. Glutathione content diminished rapidly

¹The concentrations of glutathione tested were many times that necessary to induce spawning of the swimming sexually mature males (see p. 14). Cystine of the concentration possible in a saturated state in a slightly alkaline medium, such as sea-water was not effective in stimulating either the sexual or non-sexual phase of the worms in the quiescent state.

TABLE III

EFFECT OF WATERY EXTRACTS OF TISSUES (CRUDE GLUTATHIONE) ON
INDUCING SPAWNING OF MALES OF *N. LIMBATA*

Approximately Saturated Watery Extracts of Fresh:	Glutathione* in mg. per 100 gm. Tissue	Drops Administered and Response of Male Worms
Fish muscle.....	7.5-10	1 d. spawning
Fish liver.....		1 d. spawning
Fish kidney.....		2 d. spawning
Fish blood.....		Hesitation or no effect
Cat muscle.....	Dog:76.5	1-2 d. spawning
Cat liver.....	Dog:314.0	3 d. spawning
Cat kidney.....	Dog:284.0	2 d. spawning
Cat blood.....	Dog:18.5	2 d. spawning
Cat adrenals.....	Dog:482.0	1 d. violent spawning
Cat spleen.....	Dog:210.0	1-2 d. spawning
<i>N. virens</i>	Annelids:50-75	1 d. spawning
<i>Podarke</i>	Annelids:50-75	1 d. spawning
<i>Chaetopterus</i>	Annelids:50-75	1-3 d. spawning
Coelomic fluid sea urchin.....	Echinoderms:50-75	1-3 d. slight or no effect
Yeast cake (Fleishman's).....	Yeast 340	1-3 d no effect
Cytolized <i>N. limbata</i> eggs.....	350	1-3 d. no effect

*Blanchetiere and Melon 1927, except the data concerning yeast, Fleming 1931, and *Nereis* eggs for which see p.35.

upon standing; in accord with this, extracts from aged tissues contained little or no stimulating principle. There was no evidence of greater potency of the crude glutathione extracts than of commercially obtained glutathione.

In some respects the experiments did not yield expected correlations. Liver and kidney, found to exhibit strongly positive nitro-prusside tests and recorded as possessing a much higher concentration of glutathione than muscle (Thompson and Voegtlin,

TABLE IV

EFFECT OF BOILED AND UNBOILED AQUEOUS EXTRACTS OF
AGED TISSUES ON THE SPAWNING INDUCING PROPERTY OF
COMMERCIAL GLUTATHIONE

Aqueous Extract of Aged Tissue of:	Response of Male Worms to 1-5 Drops Mixture of Aqueous Extract, Glutathione and Sea-Water	Presence or Absence of Spawning in Response to: (a) One Part 1:20,000 Glutathione, Plus (b) One Part Aqueous Extract in (c) Parts Sea-Water as Follows:					
		0	4	10	22	46	70
Beef steak:							
Unboiled....	Slight circling	++	++	++	+	±	±
Boiled.....	Circling	++	+	±	0	0	0
Veal:							
Unboiled....	No effect	+	+	+	±	0	0
Boiled.....	Circling	++	±	±	±	0	0
Ham:							
Unboiled....	Slight circling	++	++	++	++	+	+
Boiled.....	Circling	++	++	++	++	+	+
Beef liver:							
Unboiled....	Hesitation	0	±?	0	0	0	..
Boiled.....	Hesitation	+	±?	0	0	0	..
Lamb kidney:							
Unboiled....	No effect	+	±?	0	0	0	..
Boiled.....	Delayed slight circulation	±?	±?	±?	0	0	..
Beef blood:							
Unboiled....	No effect	±	0	0	0	0	..
Boiled.....		+	0	0	0	0	..
Coelomic fluid of sea urchin:							
Unboiled....	Circling	++	++	++	++	++	..
Boiled.....		++	++	++	++	+	..
Trypsin:							
Unboiled....	No effect	0	..	..	..	..	..
Boiled.....	No effect	0	..	..	..	..	..
Nereis eggs:							
Unboiled....		++	+	+	±	..	..
Boiled.....		++	+	±	±?	..	..

Control: 1:20,000 glutathione induced circling in one
part in 99 parts sea-water; i.e., 1:2,000,000 concentration.

0 = no response

± = slightest detectable movement

+

++ = strong response

1926; Blanchetiere and Melon, 1927; Wolf and Manjeau, 1933), yielded less potent extracts than did muscle. Furthermore, not only were both fresh liver and kidney low in yield of any stimulating principle, but water extracts from aged liver and kidney, and from aged blood as well, while still exhibiting a strongly positive nitro-prusside test, effected partial or even complete inactivation of the stimulating properties of commercial glutathione or of egg-water added to them. Extracts from other aged tissue had less detrimental effect. The destructive effect of these aged tissues was not due to the action of an enzyme, since the destruction occurred almost instantly and since the effect was not eliminated through first boiling the tissues (see Table IV). There is a suggestive correspondence between the aged tissues of most detrimental action and the tissues originally possessing the highest concentration of glutathione. Yeast, as obtained from yeast cake, and cytolyzed eggs are rich in glutathione and effect destruction of the spawning inducing property of glutathione or egg-water added to them. Further consideration of some of these relations will be given in Section V.

5. Glutathione Content of the Sexual and Non-Sexual Phases of Nereis Worms

Having found that both commercial glutathione and fresh aqueous extracts from certain tissues are effective in inducing spawning, an attempt was made to find if there is a possible or probable relationship between glutathione and the naturally occurring spawning inducing material of the female worm. The glutathione content was determined for Nereis eggs and the sexually mature female worm, since these represent sources of the natural spawning inducing material. For purposes of comparison, the glutathione content of spawned females and sexually mature males

of N. limbata, and of the non-sexual phase of the related species, N. virens (more readily obtained than the non-sexual phase of N. limbata), and of various marine eggs, was determined.

Tunnicliffe's iodine titration method with use of starch indicator as modified by Perlzeweig and Delrue (1927) was used. The results are given in Tables V and VI.

A much higher concentration of glutathione was found in sexually mature males and females than in the non-sexual phase of the related species of N. virens. The sexually mature limbata males contained approximately one and a half times and the similar females approximately four times, the concentration of glutathione found in the non-sexual phase of virens. The glutathione came mainly from sperm in the sexually mature males and from eggs in the ripe females. The wide variation of titration values of the spawned males or females corresponded with a wide variation in degrees of completion of spawning. The glutathione content of fresh marine eggs ranged from approximately four times that of the tissues of the non-sexual phase of N. virens in the instance of Arbacia eggs to approximately fifteen times when Asterias eggs were similarly assayed.¹ The concentration of glutathione in Nereis eggs appears to be intermediate.

In one instance, Nereis eggs, which had stood in a finger-bowl of sea-water for eight hours and appeared normal under the microscope, contained less than a fourth of the glutathione found in a similar lot of fresh eggs. Glutathione is known both to leach from detached tissues and to deteriorate upon standing.

A consideration of the above data makes clear that the potentiality of tissue to liberate the spawning inducing material

¹ See footnote to Table V and discussion, p. 44.

TABLE V

REDUCED GLUTATHIONE CONTENT OF EGGS

Date	Remarks	Vol.* in cc.	$\frac{N}{100}$ Iodine in cc.	$\frac{N}{100}$ Thio- sulfate in cc.	Net $\frac{N}{100}$ Iodine in cc.	Gluta- thione in mg. Per 100 cc. Eggs**
9/12	Nereis limbata eggs	3.30	6.00	0.10	5.90	548.8
9/12	Nereis limbata eggs	2.70	2.80	.05	2.75	312.6*
9/12	Nereis limbata eggs after eight hours in sea-water	0.80	0.35	.15	0.20	76.8*
9/3	Asterias eggs	1.35	3.20	.00	3.20	727.7
9/3	Asterias eggs	1.40	3.20	.00	3.05	668.8
9/3	Asterias eggs	2.75	6.20	.00	6.20	692.1
9/3	Asterias eggs	2.87	5.35	.00	5.35	572.3
9/12	Arbacia eggs	0.40	0.50	.17	0.33	253.3
9/12	Arbacia eggs	0.27	0.40	.05	0.35	398.0
9/12	Arbacia eggs	0.85	0.65	.05	0.60	216.7
9/12	Arbacia gonad	10.30	7.15	0.00	7.15	213.1

*The volume was determined by centrifuging except for second and third volume determinations of eggs of *N. limbata*. These two readings were made by permitting the eggs to settle in graduates and are correspondingly inclusive of a high extraneous water content.

**Ephrussi and Rapkine (1928) determined the specific gravity of sea urchin eggs to be 1.08 and the interstitial water remaining in a volume of centrifuged eggs to be 36.5 per cent. Using these values, the mgs. of glutathione per 100 gm. of eggs would be 100/68.6 times the mgs. for 100 cc. centrifuged volume. Since no determinations of relationship of gm. wt. to volume were made for the exact conditions of the present experiment, in order not to risk exaggeration, the mg. glutathione per 100 cc. and per 100 gm. are considered equivalent.

is associated with a high concentration of glutathione. Apparently there is a very great increase in glutathione content with sexual maturity. And as reported by Lillie and Just (and confirmed by the author), the ability of females of *N. Limbata* to stimulate males to spawn is only achieved with sexual maturity. The rela-

TABLE VI

COMPARISON OF THE REDUCED GLUTATHIONE CONTENT OF THE SEXUAL AND NON-SEXUAL PHASES OF NEREIS

Type of Worm Titrated for Glutathione	Number Determinations	Mg. Glutathione Per 100 gm. Worms Statistical Mean	R Value Relative to <u>N. Virens</u>
Non-sexual <u>N. virens</u> .	9	46.7 ± 2.3	
Sexually mature males <u>N. virens</u>	8	78.3 ± 4.0	6.9:1
Unspawned sexually mature females <u>N. limbata</u>	8	196.1 ± 12.8	11.6:1
Spawned sexually mature females <u>N. limbata</u>	8	77.7 ± 8.5	3.5:1

$$R = \frac{\text{Arithmetical difference of the two means}}{\sqrt{(\text{S.D.}_{m1})^2 + (\text{S.D.}_{m2})^2}}$$

Ratio of 2:1 indicates probable true difference; 3:1 indicates approximate certainty.

tively low concentration of glutathione in the non-sexual phase of N. virens and in spent females is correlated with little or no ability to induce spawning. The highest concentration of glutathione is in the eggs¹ and there is evidence that the eggs are

¹Since iodine titration here employed is based upon the general premise that glutathione is the common reducing substance of living tissue (Hopkins, 1925; Tunnicliffe, 1925), the presence of glutathione in eggs was further demonstrated. Sullivan (1926, 1929) described a distinctive test for cysteine enabling the distinction of cysteine and cystine from glutathione, and from all other substances. Glutathione upon acid hydrolysis will yield cysteine. Therefore some egg substance was tested by Sullivan's reaction before and after hydrolysis. Before hydrolysis the test was negative for cystine and cysteine and possessed a yellow color as described for glutathione. Following hydrolysis, the test gave a dark reddish wine color, indicating that a large quantity of cysteine had been freed in the hydrolysis. Glutathione was the only possible source of the liberated cysteine.

the chief source of the spawning inducing material.

6. Attempt at Correlation of Spawning Inducing
and Reducing Properties in Egg-Water

Since glutathione is present in such high concentration in eggs and is known to leach from fresh tissue, it should occur in egg-water. Utilizing very concentrated egg-water, secured by mechanically inducing several females to spawn in 5 cc. of sea-water, a reducing substance was detected by o-chlorophenyl indo-phenol (D-dye of Cohen, Chambers, and Reznikoff, 1928), and by methylene blue in a trial under anaerobic conditions in a Thunberg tube. Very few organic substances other than glutathione are able to reduce methylene blue.

The actual process of elaboration of the reduced substance from the eggs was difficult to investigate, owing to extreme dilution and possibly also to decomposition in the alkaline sea-water as rapidly, or nearly as rapidly, as elaborated. Following spawning, eggs were washed in sea-water to free them of coelomic fluid and were then allowed to stand for half an hour in a small volume of sea-water. The eggs were again washed and the water renewed. After a second interval, the new supernatant egg-water was again tested for reducing properties. In two trials,¹ reducing properties were traced to the second volume of supernatant sea-water; other trials were unsuccessful. The experiments should be continued at icebox temperature to decrease the rate of decomposition of any substance elaborated.

¹The successful trials were by use of D-dye from Dr. Cohen's laboratory; other trials by use of commercial o-chlorophenyl indophenol were unsuccessful. Any reducing substance represented in the spawning inducing material would be anticipated to be extremely dilute in consideration of the results discussed below under copper titration.

Lillie and Just (1913) found that the spawning inducing material is given off from the eggs and this was easily confirmed. The eggs continue to release the substance after repeated washings and for some hours after being shed.

In the instances where both the spawning inducing and reducing principles had been demonstrated to be present in egg-water, it was found that the reducing property remained for as long as a day after the spawning inducing property had disappeared. This would indicate either that the two properties were not associated with the same substance or that a substance possessing both properties might change in such a manner as to lose one without the loss of the other. The latter possibility is suggested by the fact that aqueous extracts of liver and kidney, upon aging, lost their spawning inducing property, and even destroyed the spawning inducing property of glutathione or of egg-water added to them, although they retained their own reducing properties. Since the reducing properties of liver and kidney persisted, it is unlikely that destruction of the reducing property of glutathione or egg-water was necessarily involved in the destruction of their power to initiate spawning. A study of the relationships presented in egg cytolysis lends further plausibility to this interpretation.

When ripe Nereis eggs were cytolized by distilled water or by mechanical injury, as by shaking the eggs with powdered glass, there was a high concentration of some reducing substance in the supernatant water. Since glutathione is present in the interior of eggs, the increase in reducing property may logically be assumed to have resulted from the freeing of glutathione. Nevertheless, the spawning inducing property of egg-water, and of commercial glutathione as well, rapidly disappeared in the presence

of cytolyzed eggs. Lillie (1919) reports an anti-fertilizin freed from the interior of eggs by cytolysis which neutralizes fertilizin.

Having found the possibility of a correlation of reducing and of spawning inducing substance in egg-water, the same was attempted for female-charged-water. Even solutions of female-charged-water which had twenty times the concentration of material necessary for one drop to induce spawning, did not possess any reducing substance detectable by any of the several tests applied. The nitro-prusside test is positive to 1:50,000 concentration of glutathione, and D-dye is sensitive to 1:200,000 concentration of glutathione. If the spawning inducing principle of female-charged-water is a substance possessing reducing properties similar to glutathione, in order that it should not be detected by D-dye in such instances as described above, its physiological potency must be ten to twenty times that of commercial glutathione. Evidence of an even greater difference in potency was obtained.

7. Evidence from Copper Titration

Copper forms very definite chemical compounds with many organic molecules and destroys the spawning inducing property of both egg-water and glutathione. A maximum dilution which would induce spawning was made of a known concentration of glutathione and of female-charged-water. Drops of 1:10,000 CuCl_2 were added to known volumes of these physiologically equivalent solutions until the spawning inducing properties of both were destroyed. In a typical comparison, it was found that 10 cc. of a solution of 1:250,000 glutathione, the maximum dilution for one drop to induce spawning of the worms used in assay, was completely inactivated by 10 drops (1/20 cc. each) of 1:10,000 CuCl_2 . The copper

concentration of the neutralized solution was approximately 1:200,000. Female-charged-water of a similar maximum dilution required only one drop of 1:10,000 CuCl_2 to inactivate 120 cc; the copper concentration was 1:24,000,000, which is 120 times as dilute as that of the neutralized glutathione solution.¹

¹Hopkins (1929) found that addition of CuSO_4 to a solution of crude glutathione resulted in the separation of a pure crystalline compound of the composition of one molecule of cuprous copper to one molecule of reduced glutathione. As the yield was not quantitative, his observation does not eliminate the consideration that other combining ratios may occur. However, there is biological evidence against any common occurrence of a multiple union of copper with one molecule of glutathione. In removing the poisoning effects of copper and heavy metals upon living substance by use of glutathione (Rapkine, 1930, 1931; Chalkley and Voegtlin, 1932), an hundred times the molar concentration of glutathione relative to the molar concentration of the poisoning metal may be required. This experimental relationship between glutathione and cell substance relative to metals would indicate that materials of the cell system poison approximately an hundred times more readily than glutathione, which is approximately the relationship above estimated.

DISCUSSION

Nereids are dioecious and characteristically dwell in individual burrows. In many species, accompanying maturation of sex products, a metamorphosis occurs adapting the worm by structure and behavior response for a wholesale exodus from the burrows and a mingling and integration of the sexes. Among such general changes there is a metamorphosis of eyespots, parapodia, musculature and frequently differentiation of body regions. In N. limbata the final changes preparatory to spawning and integration of the sexes take place only during swimming. These changes are an increase in the sensitivity of the metamorphosed chemo-receptors, a shortening of the musculature, and, in the female, the final manufacture or activation of material inducing spawning of the male.

The series of changes in structure and behavior resemble those more commonly found in the life history of insects. In functional significance the nereis phase of a swarming annelid is comparable to a larval vegetative state and the metamorphosed heteronereis phase, to an adult reproductive stage. The heteronereis phase accomplishes mingling of the sexes for reproduction and dispersal of the species. This is the same function as served by the adult stage of many insects.

In the metamorphosis preceding swarming, the males acquire a special sensitivity to glutathione not possessed by other species or by the non-sexual phase of the same species and the females undergo a large increase in glutathione content. This

suggests that glutathione, directly or indirectly, plays a significant rôle in the chemical integration originally described by Lillie and Just. At maximum sensitivity, the male may detect a concentration of 1:2,000,000 glutathione. Moreover, the males are sensitive to molecular configuration, as is indicated by their differential response to dextro- and laevo-cystine. The increase in glutathione content of the females is almost entirely due to the high concentration of glutathione in the eggs, which are the chief source of the spawning inducing material.

Glutathione and the naturally occurring material which stimulates males to spawn have the following properties in common: (1) Both are more potent at pH 6.0 than at the alkalinity of seawater. (2) Both were precipitated by acetone. (3) Both were dialyzable and filterable. (4) Both were destroyed by aging and boiling, and by irradiation with X-ray. (5) Both were more stable in acid and less stable in alkali. (6) The spawning inducing properties of both were destroyed by heavy metals, Cu, Fe, and As, and by trypsin, aged tissues, blood, sperm, and cytolized Nereis eggs. (7) Such properties of neither were affected by NaCN. (8) There is evidence that both glutathione and the spawning inducing principle are present in the cortex of the egg. An iodine avid substance exists in the cortex and the spawning inducing substance is elaborated from the surface of the egg.

However, any rôle of glutathione in the induction of spawning which may exist is not a simple one nor independent of conditioning factors. (1) The presence of glutathione does not always indicate the presence of the spawning inducing property. (a) Liver and kidney are among the richest of tissues in glutathione and do not have a correspondingly high potency in stimulating males to spawn. (b) Eggs of foreign species, Nereis sperm,

and yeast have a high glutathione content and do not induce spawning. (c) Blood, cytolyzed egg, trypsin, and aged tissues contain glutathione and not only do not stimulate spawning but destroy the spawning inducing properties of either egg-water or glutathione added to them. (2) No reducing substance can be detected in potent female-charged-water as would be expected if the natural material were glutathione as commercially obtained. (3) There is a quantitative difference between glutathione and the naturally occurring material with reference to the amount of destructive agents required for inactivation, i.e. longer boiling and longer irradiation, and more copper is required to destroy the spawning inducing property of glutathione than of physiologically equivalent concentrations of the material elaborated by female worms.

To reconcile the above two series of facts with the existence of a close relationship of glutathione to the spawning inducing material it would be necessary to assume that in the presence of certain materials, glutathione does not induce spawning, and that under certain conditions supplied in the natural state, glutathione is more potent than as obtained commercially. A short consideration justifies the former assumption. To take only one example, that of cytolyzed Nereis eggs; glutathione is indicated by the nitro-prusside and several other tests to be freed to the exterior by cytolysis and yet not only is there no increase in the spawning inducing power but this property of added glutathione is destroyed and that of added egg-water as well. Since the glutathione of the egg is not destroyed by cytolysis, it may be presumed that added glutathione is not destroyed and that the disappearance of the spawning inducing property must be due to the presence of some inhibiting material. Lillie reports an anti-fertilizin freed from the interior of eggs which

neutralizes fertilizin. It is interesting to note that the spawning inducing property of egg-water is destroyed by cytolyzed eggs and indeed by every factor tested and found to eliminate the spawning inducing property of glutathione. Therefore the presence of glutathione in the absence of spawning inducing property can no more be taken as evidence for dissociation of the natural spawning inducing material from glutathione than as evidence for a similar dissociation from Nereis egg-water.

The qualifications that the naturally occurring spawning inducing material is more readily destroyed than glutathione and that the natural material is effective in solutions exhibiting no demonstrable reducing properties would be met by establishing that under natural conditions glutathione was effective in a greater molecular dilution than under the conditions supplied by solutions of commercial glutathione. Such a condition of greater potency might result from activation or addition of a complement and be associated with species specificity. As originally described by Lillie and Just and as extended by observations of the author, other species of marine eggs tested do not give rise to material which induces spawning in males of N. limbata. As recorded in Table V, Asterias eggs are even richer in glutathione than Nereis eggs. That Asterias eggs do not give rise to a spawning inducing material, as was observed to be true, might be due to differing permeability or to a differing process of secretion. F. R. Lillie suggested that the liberation of fertilizin from eggs occurred by a process of secretion. If glutathione escaped from eggs by such a process of secretion, it would be very unlikely that it would be secreted in a pure state or that secretion from different species would be the same. It is possible that any secretion from Nereis eggs might be specialized to the advantage

of the species in a manner to contain glutathione and some specific material highly effective in inducing spawning of the males. Any secretion of eggs of other species which contained glutathione might have it in insufficient quantity to be in itself effective and lack the specific adapted complement increasing its effectiveness. Two kinds of evidence support an interpretation of secretion. (1) The spawning inducing material is not freed by cytolysis of Nereis eggs or if freed, it is masked by other substances. This would suggest a specialized rather than an accidental mode of liberation. (2) The rate of elaboration of spawning inducing material varies under differing conditions, as will be discussed further.

Although elaboration from eggs was observed to continue for a period of seven hours after being spawned, and may continue longer, the rate is a slow one and not comparable to the rate of elaboration of the spawning inducing material by the swimming female, which may continue for thirty-six hours. That the swimming induces a manufacture of the spawning inducing material rather than merely mechanically frees any accumulation of material from the worms is indicated by the fact that the actual amount of material present in certain observed worms was greater after swimming than the amount in similar control worms remaining quiescent (see Table I and Graph II). That the eggs are actually involved in the manufacture of the spawning inducing material is proven by the fact that spent swimming females do not elaborate any appreciable amount of the material.

The interval of swimming of an unspawned female required for supplying optimum conditions for elaboration of material by the eggs may be noted to vary with the physiological state of the worms. Relatively fresh worms usually reach a maximum in three

to four hours as compared to twelve hours for worms which have been aged in an icebox for a week or longer. An interpretation of a secretion process as the source of the naturally occurring spawning inducing material gives plausibility to the hypothesis that some activation or addition of a complement to a glutathione compound may occur.

It may be remembered that l-cystine is only one-tenth or less as effective in inducing spawning as glutathione and that d-cystine is only one-tenth or less as effective in inducing spawning as l-cystine. Copper titration indicated that the natural material may be a hundred times as effective as glutathione. If the sensitivity of the sense organs of the male worm to difference in molecular configuration is proportionate, one might expect the naturally occurring material to differ from glutathione by a molecular configuration somewhat greater than the difference in configuration of glutathione and l-cystine, or l-cystine and d-cystine.

Another paper will concern itself with the relation of the spawning inducing material and fertilizin, which was suggested in the original work of Lillie and Just. The properties of the spawning inducing material as discussed in this paper are similar to those of fertilizin with respect to neutralization by cytolized egg, blood, and sperm, and destruction by copper. However, the spawning inducing material was ascertained to be dialyzable as contrasted to fertilizin as described by Lillie.

SUMMARY

- (1) The spawning mechanism of N. limbata appears to involve four component muscle actions: (a) ventral then (b) dorsal contraction, (c) lateral sinusoidal movements coordinated with parapodial beating, and (d) a peristalsis progressing anteriorly. These components would tend to place the inter-parapodial regions under strain, and, at the same time, place the internal contents under pressure.
- (2) The cerebral ganglion is necessary to orientation of the worm to the source of chemical stimulation and for the circling response. The suboesophageal ganglion is essential to swimming. There is some evidence that the giant fibers may function in swimming.
- (3) At sexual metamorphosis a sense enabling spacial orientation of the swimming worms becomes more sensitive. The sense organs involved are located on the parapodial cirri. In the male at sexual metamorphosis, the region of the epitoke becomes especially sensitive to a constituent of egg-water, and also to glutathione. The sensitivity is greater in the swimming than in the quiescent worm.
- (4) The spawning response of the male is a typical neuro-sensory response, but the response of the female has some characteristics of a hormonal response. A neuro-hormonal mechanism is suggested. There is evidence that after prolonged stimulation, the typical nervous response of the male worm may assume hormonal characters.

- (5) The swarming of the heteronereis phase of N. limbata may be induced in the laboratory by artificial darkness following light. In the laboratory, once in the swimming state, the worms will swim indefinitely in any illumination unless a suitable tactile stimulus is encountered; this induces quiescence almost instantly. It is probable that tactile stimuli are of supplementary importance in the rhythms occurring in nature.
- (6) Swimming is not only necessary to the normal spawning reaction of N. limbata, but is necessary for any high degree of sensitivity to egg-water and is entirely necessary for elaboration of any chemical stimulating to the opposite sex. In the quiescent state, males and females may remain indefinitely in close proximity without reciprocal stimulation or spawning.
- (7) Glutathione in concentrations of 1 to 10 parts per million added to a vessel of sea-water containing swimming males will induce spawning. The induced reaction is entirely characteristic and indistinguishable from that initiated by water charged by a swimming female worm or by supernatant egg-water.
- (8) The sexually mature females contain about four times the concentration of glutathione characteristic of the closely related species, N. virens. The eggs are the chief source of the high concentration of the sexually mature female. The spawned female is not rich in glutathione and does not give rise to an effective amount of the spawning inducing substance. The eggs elaborate the spawning inducing material even after repeated washings in sea-water. A reducing substance ~~was~~ present in concentrated egg-water, but was not demonstrated in physiologically potent female-charged-water.

- (9) The properties of the spawning inducing principle of egg-water and a physiologically equivalent solution of glutathione are in qualitative but not necessarily quantitative agreement with respect to: precipitation by acetone, dialyzability, destruction upon standing in sea-water at room temperature, destruction by boiling in sea-water, relative stability in acid solutions and relative instability in alkaline solutions, destruction by irradiation, destruction by copper and arsenic poisoning, lack of destruction by NaCN, inactivation by stale blood and cytolyzed egg, and both are rendered less effective in the presence of sperm. In the presence of certain substances neither glutathione nor egg-water induce spawning.
- (10) Copper titration of female-charged-water and of glutathione, utilizing complete loss of spawning inducing properties as an end point, indicate that the naturally occurring material in egg-water may be effective in a hundred times or more the molecular dilution necessary to constitute an effective concentration of glutathione.
- (11) The ability of the specialized chemo-receptors, distinctive to the male Nereis worm, to react to a common molecular configuration as represented in glutathione and cystine though reacting differentially, and to distinguish laevo- and dextro-cystines, gives evidence of a sensitivity of these organs to specific molecular configuration. It is probable that glutathione shares, in part, the molecular configuration of the naturally occurring spawning inducing substance to which the sense organs are adapted. Some activation of glutathione, or addition of a complement, may constitute the naturally occurring molecular configuration.

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PLATE I

ROLE OF A VIBRATORY SENSE IN ORIENTATION
IN CIRCLING

- A. Male worms swimming oriented to sides of a bowl.
- B. The above worms swimming oriented to a few introduced spontaneously circling males.
- C. Another view of B.

PLATE I



A



B



C

PLATE II

NORMAL AND ARTIFICIALLY INDUCED SPAWNING

- A. Male and female worms swimming in normal "around the dish" pattern prior to reciprocal spawning stimulation.
- B. Male worms circling about a spawning and circling female, i.e., normally induced spawning reaction of males.
- C. Male worms in normal "around the dish" swimming without stimulation of glutathione.
- D. Male worms circling about a drop of 1:1,000,000 glutathione, i.e., artificially induced spawning of males.

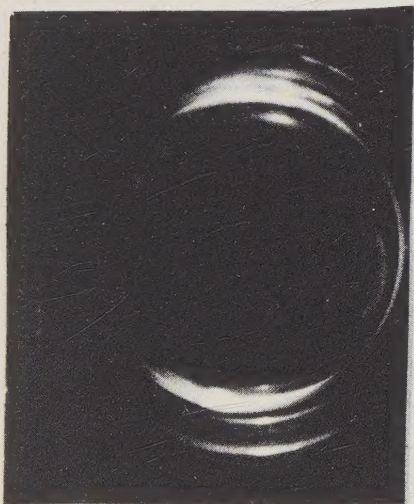
PLATE II



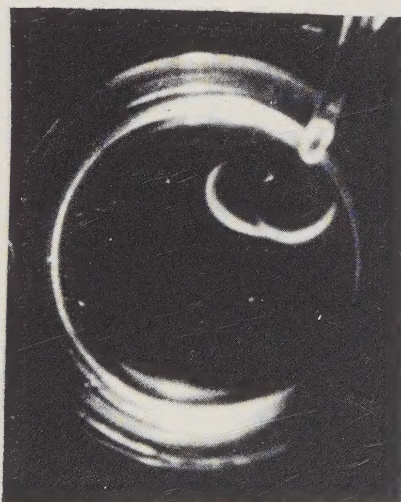
A



B



C



D

